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Lovastatin stimulates human vascular smooth muscle cell expression of bone morphogenetic protein-2, a potent inhibitor of low-density lipoprotein-stimulated cell growth☆

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Abstract

Bone morphogenetic proteins (BMPs) stimulate ectopic bone formation in skeletal muscle. Here we show that human vascular smooth muscle cells (VSMC) abundantly express mRNA encoding for BMP receptor type II, BMP-2, and BMP-7 proteins. Treatment with the 3-hydroxy-3-methylglutaryl coenzyme A inhibitor lovastatin (34 μM) increased BMP-2 gene transcription >14-fold as measured by real-time PCR analysis ($P < 0.05$ vs. solvent control). Moreover, VSMC proliferation stimulated with native low-density lipoprotein (100 μg of protein/mL) was prevented by either human recombinant BMP-2 or BMP-7 at concentrations of 100 ng/mL ($P < 0.05$). Both BMPs also inhibited basal cell proliferation ($P < 0.05$). Induction of BMPs and subsequent inhibition of VSMC growth and/or induction of vascular bone formation could contribute to the mechanisms by which statins increase plaque stability in patients with coronary atherosclerosis.

Keywords: BMP-statins; HMG-CoA reductase inhibitor; Low-density lipoprotein; Thymidine incorporation; Plaque rupture; Bone; Risk factors; Reverse transcriptase polymerase chain reaction; Cholesterol; Transforming growth factor-β

Elevated LDL cholesterol levels are a major risk factor for atherosclerosis [1] which is characterized by chronic inflammation, vascular accumulation of low-density lipoproteins, and growth of vascular smooth muscle cells, resulting in vascular plaque formation [1,2]. The advanced atherosclerotic plaque contains a lipid core covered by a fibrous cap which, if ruptured, frequently leads to myocardial infarction [3,4]. Although some vascular cells have osteoclastic activity [5] and calcification as well as cartilage and bone formation occurs in advanced atherosclerosis [6–8], the regulation of these processes is still poorly understood.

LDL receptor-related proteins have been recently implicated in bone formation [9,10], suggesting a link between lipids and osteogenic cellular activities. Bone morphogenetic proteins (BMPs) were initially isolated from bone matrix and can induce ectopic bone formation in tissues such as skeletal muscle [11]. The BMP family consists of approximately 20 isoforms which bind to specific receptors and belong to the transforming growth factor-β superfamily [12]. BMPs and BMP receptors contribute to vasculogenesis [13] and have been detected in intact as well as in atherosclerotic vascular tissue [6,14,15]. Conflicting reports on the effects of BMP-2 on vascular smooth muscle cell growth have been published [14]; similarly, inhibitory effects on PDGF-stimulated vascular cell growth [16] as well as lack of effects [17] have been described using BMP-7.

In the vasculature, HMG-CoA reductase inhibitors (statins) alter vascular matrix composition resulting in atherosclerotic remodelling and increased plaque stability [4,18,19]. Lovastatin was identified as a potent

☆ *Abbreviations:* BMP, bone morphogenetic protein; mRNA, messenger ribonucleic acid; RT-PCR, reverse transcriptase polymerase chain reaction; LDL, low-density lipoprotein; HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A; VSMC, vascular smooth muscle cells.

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inductor of BMP-2 promoter activity in vitro and shown to stimulate bone formation in vivo [20]. Induction of BMP-2 expression by statins also occurs in murine embryonic stem cells [21]. In the present study, we investigated whether human vascular smooth muscle cells (VSMC) express BMP-2, BMP-7, and the BMP type II receptor, and whether lovastatin modulates BMP-2 gene expression in these cells. We also determined the effects of human recombinant BMP-2 and BMP-7 on VSMC proliferation stimulated with low-density lipoprotein (LDL).

Materials and methods

Materials. Smooth muscle cell growth medium consisted of DMEM and Ham's F-10 medium (1:1, v/v) (Bioconcept, Allschwil, Switzerland) and 10% fetal calf serum (FCS, Sigma Aldrich, Germany). Recombinant human BMP-2 and BMP-7 (R&D Systems, UK) were dissolved according to manufacturer's instructions. Lovastatin (Sigma, Switzerland) was dissolved in 0.1% DMSO. PCR probes were obtained from Qiagen-Operon (Cologne, Germany) and PCR primers were synthesized at Microsynth (Balgach, Switzerland).

Preparation of low-density lipoprotein. Low-density lipoprotein (LDL) was isolated from pooled human plasma. Freshly drawn EDTA blood from normolipemic donors taking no medication was obtained at the blood bank of our institution. Plasma was subjected to ultracentrifugation in the presence of 1 mM EDTA in a KBr gradient at 200,000*g* for 14 h at 4 °C as described in [22]. The LDL fraction was decanted and dialysed at 4 °C for 24 h against three changes of 1500 ml of 150 mM NaCl and 0.25 mM EDTA at pH 7.4. After dialysis, LDL was concentrated using membrane separation (Centrisart 1 tubes; Sartorius, Göttingen, Germany) with a cutoff at 20 kDa. LDL was sterilized using 0.22 mm Millipore filters and stored in the dark at 4 °C. Concentration of LDL was calculated as μg of protein measured by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA) [23].

Human vascular smooth muscle cell culture. Experimental procedures were in accordance with institutional guidelines. Human umbilical cords were obtained at the Department of Obstetrics and Gynecology at the University Hospital Zürich. Umbilical vein smooth muscle cells were isolated using the explant technique as described and cultured in 75 cm^2 culture flasks (Falcon), using smooth muscle cell growth medium [22]. Cells were characterized as smooth muscle cells by their hill- and valley morphology by phase-contrast microscopy and positive staining for smooth muscle cell-specific α-actin by immunofluorescence [24]. Cells were passaged after treatment with 0.05% trypsin/0.02% EDTA in phosphate-buffered saline. Subconfluent cells of passages 3–5 obtained from 8 different umbilical chords were used for experiments.

Experimental protocols. For cell proliferation experiments, subconfluent VSMC grown under standard conditions were serum-starved (0% FCS) for 24 h to induce quiescence and treated with BMP-2 (100 ng/mL), BMP-7 (100 ng/mL), or native LDL (100 μg/mL) in the absence of serum. In experiments to determine the effects of BMPs on LDL-induced cell proliferation, quiescent cells were pretreated with BMPs 3 h before native LDL (100 μg/mL) was added. Cell proliferation was measured by [^{3}H]thymidine incorporation as described in [22]. Briefly, after 19 h of incubation, 3 μCi of [methyl-^{3}H]thymidine (1.5 μM) was added and cell proliferation was assessed after 24 h by aspirating the medium and subjecting the cells to DNA extraction as described in [22].

For expression experiments, the optimal dose of lovastatin (34 μmol/L) was determined by dose-finding experiments by investigating the effects on cell proliferation after stimulation with 2.5% fetal calf serum. Cells were harvested under unstimulated conditions (serum-starved for 24 h in the presence of solvent control) or after treatment with lovastatin at different time points (3, 6, 12, 18, and 24 h) as described previously [25]. Total RNA was extracted as described below and RT-PCR was performed [25].

Real-time polymerase chain reaction. Total RNA was extracted from vascular smooth muscle cells using the silica-based RNeasy method (Qiagen, Hilden, Germany). Purity of RNA was determined photospectrometrically (ratio 260/280 nm), by gel electrophoresis, and by RT(−) reactions (PCR with non-transcribed RNA). For each sample, 400 ng RNA was reverse transcribed (Omniscript RT kit, Qiagen, Hilden, Germany). Real-time quantitative PCR was used to determine the expression of genes encoding for human bone morphogenetic protein-2 (NM_001200), human bone morphogenetic protein-7 (NM_001917), and human bone morphogenetic protein receptor type II (NM_033346).

Gene expression was calculated using the $\Delta\Delta_{CT}$ method [25]. Efficiency (E) was calculated from the slopes (s) of the standard curve for each gene according to the formula $E = 10^{-1/s}$ [26]. PCR were run on the iQ iCycler (Bio-Rad), using specific cDNA primers and probes. Optimization of PCR was performed until the efficiency of the PCR was >0.99 and <1 (slope value of 3.3–3.39). Two-step PCR was performed with Quantitect probe PCR kit (Qiagen) as follows: activation of the hot start *Taq* polymerase for 15 min (95 °C), followed by 50 cycles of denaturation at 95 °C for 30 s (step 1), and annealing and extension for 60 s at 60 °C (step 2). Fluorescence was detected at the end of each extension step.

The following sets of primers and probes were used: 5′-GGA GAA GGA GGA GGC AAG-3′ (forward), 5′-GAC ACG TCC ATT GAA AGA GC-3′ (reverse), and [6∼FAM]-AGG AAC GGA CAT TCG GTC CTT GC [TAMRA-FAM] (probe) for amplification of a human bone morphogenetic protein-2-specific cDNA fragment; 5′-CGG ACT CGT TTC CAG AGG TAA TT-3′ (forward), 5′-GGT CAA TTT TCC TTT CGC ACA GA-3′ (reverse), and [6∼FAM]-ACC AAT GTG CCC ACC CCT TGC CA [TAMRA-FAM] (probe) for a human bone morphogenetic protein-7 cDNA fragment; 5′-GAT GGC AAA TCA GGA TCA GG-3′ (forward), 5′-CCT CAC AGT CCA GCA ATT CAG-3′ (reverse), and [6∼FAM]-CTC CCT ATT CTC TCT TAA GCG GTG GCG [TAMRA-FAM] (probe) for amplification of a human bone morphogenetic protein receptor type II-specific cDNA

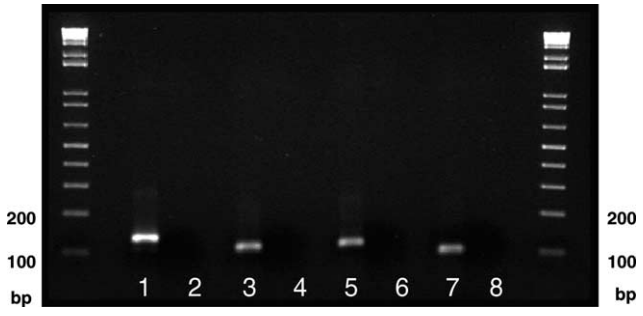

Fig. 1. Messenger RNA expression of β-actin, BMP-2, BMP-7, and BMP receptor type II. Total RNA was extracted from quiescent cultured human vascular smooth muscle cells and RT-PCR was performed with specific primers and visualized using gel electrophoresis using the GelDoc system (Bio-Rad). Shown here are representative results of four independent experiments demonstrating abundant expression of all genes under basal conditions. Bands were detected at the expected size: lane 1, β-actin (134 bp); lane 2, β-actin negative control (H_2O); lane 3, BMP receptor type II (115 bp); lane 4, BMP receptor type II negative control (H_2O); lane 5, BMP-2 (100 bp); lane 6, BMP-2 negative control (H_2O); lane 7, BMP-7 (117 bp); and lane 8, BMP-7 negative control (H_2O). Ladder depicts molecular weight marker (1 kb plus DNA ladder, Invitrogen).

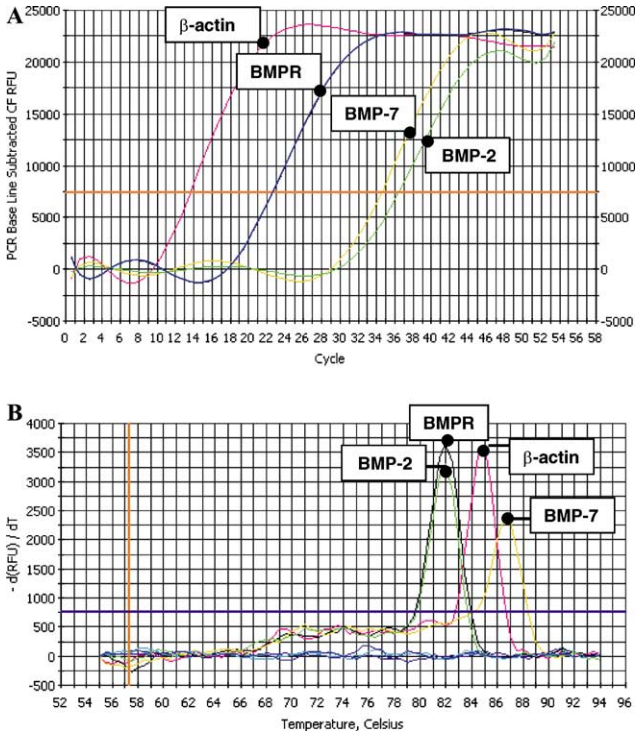

Fig. 2. Messenger RNA expression levels of BMP receptor type II and BMPs. (A) Real-time analysis of the gene expression of β-actin, BMP receptor type II, BMP-2, and BMP-7. BMP receptor gene was expressed at a much higher level than the genes of both BMP proteins. On the *x*-axis CT values are given (a Δ_{CT} of 3.33 represents a 10-fold expression difference). Original recording of one of the four experiments performed on the iQ iCycler. (B) PCR-based melting curve analysis of the four genes examined, demonstrating a lack of primer-dimer or unspecific amplicon formation during the PCR.

fragment. Human β-actin (primers 5′-CCA CGA AAC TAC CGT TAA CTC C-3′ (forward), 5′-CAG TGA TCT CCT TCT GCA TCC-3′ (reverse), and [6∼FAM]-TGA AGT GTG ACG TGG TGG ACA TCC GC [TAMRA-FAM] (probe) was used as a house-keeping control. Identity and specificity of amplicons was confirmed by agarose gel electrophoresis (Fig. 1), melting curve analysis using the Quantitect SYBR green PCR kit (Qiagen) (Fig. 2B), and sequencing (Microsynth, Balgach, Switzerland).

Calculations and statistical analyses. All data are means ± SEM. For comparison of group means, one-way ANOVA or the Mann–Whitney *U* test was used as appropriate. Significance was accepted at *P* values below 0.05.

Results

Messenger RNA expression of BMP type II receptor, BMP-2, and BMP-7

Using specific primers and the sensitive RT-PCR technique, we show that genes encoding for BMP-2, BMP-7, and the BMP type II receptor are abundantly expressed in cultured human umbilical vein VSMC (Fig. 1). Quantitative PCR analysis showed that messenger RNA for the BMP type II receptor was expressed at a more than 1000-fold higher level than both BMP proteins (Fig. 2A, representative of four independent experiments).

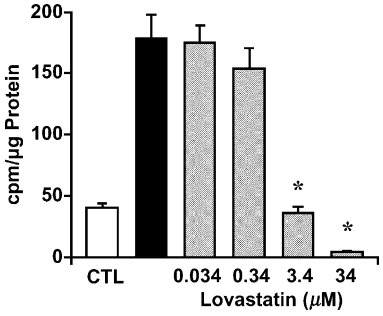

Fig. 3. Concentration-dependent inhibition of FCS-induced VSMC proliferation by lovastatin. Vascular smooth muscle cells stimulated with FCS (2.5%) were treated with increasing concentrations of lovastatin (0.034–34 µM, dissolved in DMSO, hatched bars) or vehicle control (DMSO, CTL, open bar) for 24 h, $n = 4$ for each treatment group. Error bars represent standard error of the mean. $^*P < 0.05$ vs. FCS (filled bar). Error bars represent standard error of the mean.

Lovastatin induces BMP-2 mRNA expression

Lovastatin dose-dependently inhibited cell proliferation stimulated 2.5% FCS, inhibition was almost complete at 34 µmol/L (Fig. 3) and this dose was used for subsequent expression experiments. Treatment of quiescent vascular smooth muscle cells with this concentration of lovastatin was performed at different time points and resulted in a robust, more than 14-fold stimulation of BMP-2 mRNA expression after 18 h ($P < 0.05$ vs. solvent control, Fig. 4) The stimulatory effect of lovastatin on BMP-2 mRNA expression was seen as early as 3 h after treatment and was still present at 24 h after stimulation (data not shown).

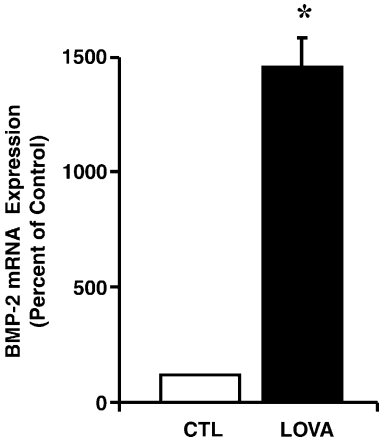

Fig. 4. Effect of lovastatin on VSMC gene expression of bone morphogenetic protein-2. Quiescent vascular smooth muscle cells were treated with lovastatin (34 µM, dissolved in DMSO, Lova) or vehicle control (DMSO, CTL) for 18 h. Data of four independent experiments are shown, PCRs were performed in triplicate. $^*P < 0.05$ vs. solvent control. Error bars represent standard error of the mean.

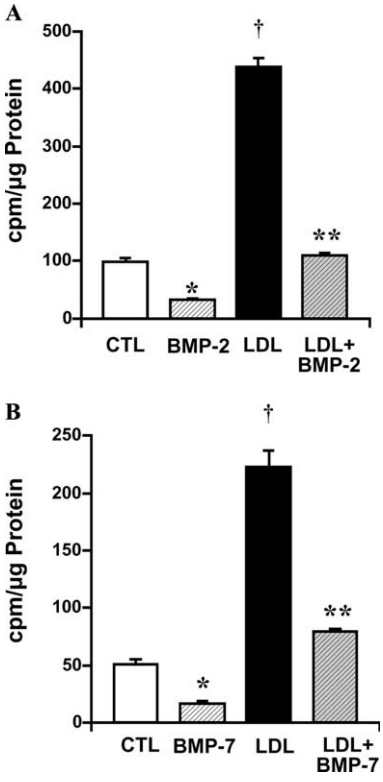

Fig. 5. Human recombinant BMP-2 and BMP-7 prevent human VSMC proliferation induced by native low-density lipoprotein. Quiescent vascular smooth muscle cells were treated with 100 ng/mL of either BMP-2 (A) or BMP-7 (B) in the absence or presence of native LDL (100 μg/mL) or vehicle control ($n = 6$ for each treatment group). Representative results of at least four independent experiments for either BMP-2 or BMP-7 are shown. *$P < 0.05$ vs. solvent control; †$P < 0.05$ vs. LDL. Error bars represent standard error of the mean.

Inhibition of LDL-stimulated VSMC proliferation by BMP-2 and BMP-7

Preliminary experiments showed little or no effect of BMPs on cell proliferation at concentrations below 1 ng/mL. We used human recombinant BMP-2 or BMP-7 at concentrations of 100 ng/mL to investigate the effects on native LDL-stimulated and basal VSMC proliferation as measured by [^{3}H]thymidine incorporation. Native LDL caused an approximately 4-fold increase of cell proliferation, which was abrogated by both BMP-2 (Fig. 5A) and BMP-7 (Fig. 5B, both $P < 0.05$ vs. LDL). Both BMPs also inhibited cell proliferation under basal conditions (Fig. 5, open hatched bars, $P < 0.05$ vs. basal).

Discussion

In this study, we show that in human vascular smooth muscle cells lovastatin strongly induces BMP-2 gene expression and that BMP-2 and BMP-7 prevent LDL-induced cell proliferation. We further show that these cells abundantly express messenger RNA of the BMP type II receptor as well as BMP-2 and BMP-7 at different expression levels.

BMP-2 has been detected in calcified human atherosclerotic lesions and in fibrous cap intimal VSMC of the atheromatous plaque whereas in the same studies no BMP-2 was detected in intact arterial tissue [6,15]. As shown in the present study, the expression level of both BMP-2 and BMP-7 transcripts in human VSMC is low compared to that of the BMP type II receptor (Fig. 2A). Thus, it is reasonable to speculate that in normal arteries sensitivity of the methods used previously [6,15] was not sufficient to detect BMP-2 either at the mRNA or protein level. In the present study we show abundant expression of BMP-2 in human umbilical vein VSMC, supporting observations by Willette et al. [14]. These investigators also described constitutive expression of the BMP-2 gene as determined by RT-PCR in different types of human arterial vascular smooth muscles [14]. BMP-2 inhibits rat aortic VSMC proliferation stimulated with serum or transforming growth factor β-1 [17]. In line with our observations in human vascular smooth muscle, Nakaoka et al. also showed that BMP-2 inhibits thymidine incorporation in rat aortic smooth muscle cells under basal conditions [17]. Willette et al. [14] observed a strong stimulating effect of low concentrations of BMP-2 on human aortic vascular smooth muscle cell migration. In contrast to our observations and those by Nakaoka et al. [17], these investigators were unable to demonstrate effects of BMP-2 on either basal or PDGF-stimulated cell growth [14]. The lack of inhibitory effect on cell proliferation could most likely be explained by the BMP-2 concentrations used, which were much lower than those used in our study and by Nakaoka et al. [17] and also did not show any effect in our experiments.

We now show that the proliferative effects of native LDL, a potent stimulus of cell proliferation in human vascular smooth muscle cells, which activates redox-sensitive pathways [22], can be blocked by either BMP-2 or BMP-7. These findings suggest that BMPs can interfere with this specific type of stimulated cell growth by yet unknown mechanisms. Redox-sensitive regulation of proteins involved in bone formation has been reported [26,27] and recent work from our laboratory showed that native LDL is capable of rapidly increasing the production of ROS and ERK1/2 activation [22]. Furthermore, we have shown that antioxidants can interfere with native LDL-induced cell growth [22]. The finding that both BMP-2 and BMP-7 were as efficient as antioxidants to prevent LDL-stimulated cell proliferation [22] suggests the possibility that redox-sensitive mechanisms are involved. Though low-density lipoproteins and/or related receptor proteins have been recently implicated in vascular osteoblastic activity and cell differentiation [10,26,28], it remains to be established

whether LDL is directly involved in such phenotypic modulation of vascular smooth muscle.

In patients with atherosclerosis, statins reduce cardiovascular events and increase plaque stability [4], effects that cannot be solely attributed to the lipid-lowering properties of these drugs [4,29]. Previous observations made in bone tissue [20] and in murine embryonic stem cells [21] have shown that statins can induce BMP-2 expression. Our data extend these observations to human vascular smooth muscle and suggest the possibility that statins can promote a pro-osteogenic phenotype in human arteries. Currently it is not clear whether statins modulate bone growth in the vasculature of patients with atherosclerosis. Vascular bone formation is absent in intact arteries, whereas bone formation and upregulation of BMP-2 occur in certain areas of atherosclerotic lesions [6,15]. Thus, changes in BMP-2 expression and activity could possibly play a role for plaque stability in vascular tissue of patients with atherosclerosis receiving statins.

In contrast to BMP-2, the vascular actions of BMP-7 have been less investigated. In the present study we demonstrate constitutive gene expression of BMP-7 in human vascular smooth muscle cells. We also show that BMP-7 strongly inhibits basal and LDL-induced VSMC proliferation. To the best of our knowledge, this is the first study demonstrating that BMP-7 is constitutively expressed in human umbilical vein vascular smooth muscle. Our data on cell proliferation contrast previous work by Nakaoka et al. [17] who did not observe inhibitory effects of BMP-7 on the basal proliferation of rat aortic VSMC. Dorai et al. [16] reported inhibitory activity of BMP-7 in quiescent VSMC and in cells stimulated with PDGF or TGF-β, an effect that was less pronounced than the effects of BMP-7 on LDL-stimulated cell growth in our study. Since in our study the magnitude of inhibitory effects was similar between BMP-2 and BMP-7 and both BMPs were used at the same concentration, these proteins may share a common mechanism of actions.

Taken together, we have shown potent induction of BMP-2 gene expression by lovastatin in human vascular smooth muscle cells and strong growth inhibitory effects of BMP-2 and BMP-7 both in cells stimulated with native LDL and under basal conditions. The inhibitory effects on LDL-induced cell growth suggest therapeutic potential of BMPs for the treatment of atherosclerosis, whereas induction of BMP-2 could play a role for the beneficial changes in vascular matrix formation seen in patients with atherosclerosis receiving statin therapy.

Acknowledgments

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